

QUICK PCR TO DETECT *M. TUBERCULOSIS* AND *M. BOVIS* IN SWINE BLOOD SAMPLES

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Abstract The disease control strategies in livestock are the key areas to enhance improvement of livestock production and safety of livestock product consumers. Therefore, reliable techniques for quick detection and specific identification of disease causing agents need to be developed.

In this study a nested PCR was developed using primer primer 5.0 software for primer design targeting IS1081 gene conserved regions in both *M. tuberculosis* and *M. bovis*. Two sets of primers TB-Q1/TB-Q2 and TB-B1/TB-B2, were designed to detect *M. tuberculosis* and *M. bovis* DNA extracted from blood samples. The findings were, the assay is sensitive enough to detect up to 1.35fg of the antigen DNA with 100% specificity. When compared with bovine Purified Protein Derivative skin test, it had better results in terms of sensitivity and specificity. Furthermore, an OxyR gene was targeted for another set of primers OxyRMT-2.1/OxyRMT-1 specific for *M. tuberculosis* while OxyRMT-2.1/OxyRMB-2 for *M. bovis*.

Introduction Most developing countries rely on livestock activities, but have a lot of shortcomings in implementing effective disease control programs, because of poor infrastructures (Blood *et al*, 1988). This is a hindrance for export of livestock and their products because the office Internationale des Epizooties (OIE) regulations requires that an exporting country should be free from certain diseases (OIE, 1994).

The field trials of this research were done on a farm in Guangdong Province, China. As of 2001, China had a national inventory of pigs estimated at 454 million herds, as compared to that of the world of 928 million (Storburg *et al*, 2001). The routine tuberculin test has been used with satisfactory results in other parts of the world (Noordhuizen *et al*, 1997), but not enough information is available for its use in Guangdong.

The fact that there is a gradual increase in bovine TB in England among the cattle herds which were previously declared free from TB with Tuberculin skin tests (British Veterinary Association, 2005), calls for modern diagnostic tools in order to control and eradicate TB in livestock and humans.

The definitive diagnoses of Tuberculosis (TB) have been the culture and isolation of the bacteria (Parsons *et al*, 2002). But the direct smear microscopy has low sensitivity and does not differentiate the *M. tuberculosis* complex (TBC). On the other hand, culture and species assignment, even when carried out, are time-consuming and not accurate confirmatory tests (Collins *et al*, 1984; Kamerbeek *et al*, 1997). The skin test has a low sensitivity of about 65.6% to 70 % (Niemann *et al*, 2000).

The objective of this research was to target IS1081 gene sequence and develop a quick PCR protocol which could be applied in swine to diagnose *M. tuberculosis* and *M. bovis*. Furthermore, to validate it by carrying out comparative studies with other tests, and to clone and sequence some detected DNA fragments for the basis of further research.

Materials and Methods The *M. tuberculosis* and *M. bovis* used in this study were from the National Institute for Pharmaceutical and Biological products, China. Whereas, *Salmonella*, *Pasteurella multocida*, *Streptococcus*, *Actinobacillus pleuropneumoniae* and *Staphylococcus* all came from our laboratory.

Genomic DNA was extracted as follows; 300µL of blood sample was pipetted into the 1.5mL vial. Then 30µL of 10% (w/v) SDS (sodium dodecyl sulfate) was added to lyse the bacteria. The samples were incubated at room temperature for 15 minutes and then washed with 1mL of double distilled water (ddH₂O) while being centrifuged at 10,000 rpm, the supernatant was discarded and then 1mL of ddH₂O added and centrifuged again at 10,000 rpm. The supernatant was discarded and 50µL of TE (10mMTris, 1mM EDTA, pH 8.0) was added and the vials incubated at 100°C for 10 minutes and then centrifuged for 15minutes at 15,000rpm. The supernatant was the DNA.

The IS1081 from the GeneBank was used for the primer design for *M. tuberculosis* and *M.*

bovis, while the OxyR gene derived primers for differentiating them. The primers were selected by importing the sequences into primer premier 5.0 program at www.premierbiosoft.com/primerdesign/primerdesign. The sequences of the primers used in the assay are: the primer pairs that enable identification of *M. tuberculosis* and *M. bovis*; TB-Q1/TB-Q2 and TB-B1/TB-B2; for the differentiation of *M. tuberculosis* and *M. bovis* oxyRTB-2.1/oxyRMT-1, oxyRTB-2.1/oxyRMB-1 (Sreevatsan *et al*, 1996; Espinosa de los Monteros *et al*, 1998).

PCR conditions were optimized with respect to annealing temperatures and the number of amplification cycles. A 25µL reaction mixture consisted of purified genomic DNA template from the sample, 20pM for all the primers, 10x PCR buffer, 2.5mM dNTP, 5% DMSO (dimethyl sulfoxide), Taq enzyme. The samples were amplified using a thermal cycler (Whatman Biometra T-gradient PCR machine)

After looking at the different annealing temperatures in the gradient reactions and adjusting the reaction times, a final 30 cycle PCR protocol was developed consisting of a pre-denaturing temperature at 94°C for 8mins, denaturing at 94°C for 40sec, annealing at 57°C for 40sec, extension at 72°C for 45sec and final extension at 72°C for 7min. Using the primers amplifying 476bp and DNA templates, a 25µL PCR protocol reaction was set up.

TB-Q1Forward: 5'CGAAGGAAATGACGCAATGA3'

TB-Q2Reverse: 5' CATCTCCGCAAAGCCTGGG 3'

The reaction mixture was: 2µL DNA template, ddH₂O, 10 x buffers, 2.5mM 4xdNTP, 20µM Primers 1&2, 2.5U Taq DNA polymerase.

Using a similar approach, a second 30 cycle nested PCR protocol was developed consisting of a pre-denaturing temperature at 94°C for 6min, denaturing at 94°C for 30sec, annealing at 67°C for 30sec, extension at 72°C for 40sec and final extension at 72°C for 8min. The second set of primers:

TB-B1 Forward: 5' ACAGGCGAGCCCGGATCTGCTG 3'

TB-B2 Reverse: 5' GTTCAGCTCGCTTGCGGCGCTG 3'

amplifying 248bp was used and 1µL of the PCR product from the first PCR protocol was used as a DNA template. In a similar way, a 25µL nested PCR protocol reaction was developed. The reaction mixture was: DNA template, ddH₂O, 10 x buffers, 2.5mM 4xdNTP, 20µM Primers 1&2, 2.5U Taq DNA polymerase.

A similar reaction mixture was established for the differentiation of *M. tuberculosis* and *M. bovis* with primers amplifying 270 bp.

Forward: OXYRMT-2.1: 5' TGGCCGGGCTTCGCGCGT 3'

Reverse: OXYRMT-1: 5' GCACGACGGTGGCCAGGCA 3'

Reverse: OXYRMB-2: 5' TGCACGACGGTGGCCAGGTA 3'

After extensive adjustments the following conditions were optimal; the initial DNA denaturation was 94°C for 8min, in cycles denaturation was 94°C for 45sec, the annealing temperature was 71°C for 30sec, and the extension was done at 71°C for 1min with the final extension at 72°C for 7min.

To determine the detection limit of this assay, a solution with the DNA from the reference strains of *M. bovis* and *M. tuberculosis*, were serially diluted into 13.5ng, 1.35ng, 135pg, 13.5pg, 1.35pg, 135fg, 1.35fg, 0.135fg and used as templates in the assay. The detection limit of the assay was defined as the lowest concentration of DNA that could be amplified and visually detected.

Comparative tests were done with Purified Protein Derivative tuberculin (PPD) of bovine origin and of avian origin (0.1 mL of 20,000IU of PPD).

Five different DNA fragments (TB-Q1, TB-Q2, D2 E2 and F2) were cloned into a pMD18-T vector to make recombinant plasmids. They were later sequenced and the sequenced data organized and edited electronically with the EDITSEQ, ALIGN and MEGLIGN PROGRAMS using computer software DNASTAR at www.dnastar.com and then compared with the IS1081 published in GeneBank.

Results We varied the annealing temperatures between 53°C and 73°C and the number of cycles between 25 and 35 for each PCR assay. The optimal annealing temperatures were 57°C for 40 seconds, 67°C for 30 seconds and 71°C for 30 seconds for the first and second nested PCR assays for detection of *M. tuberculosis* and *M. bovis* and for differentiating them, respectively. The optimal numbers of PCR cycles were 30 in all the three assays. The assay could detect 1.35pg but improved 1000 times when nested PCR was used, to detect up to 1.35fg as shown in figure 1.

The tests with: *salmonella*, *Pasteurella multocida*, *streptococcus*, *actinobacillus pleuropneumoniae*

moniae and *staphylococcus* had no false-positives or false-negatives. This assay was much sensitive and specific compared to bovine PPD, Table 1

The PCR of recombinant plasmids (REA), gave the expected 248bp. For the Restrictive Endonuclease Analysis (REA), the expected 2940bp band was observed for a single enzyme analysis (Hind III), whereas two bands 248 and 2692bp for double enzyme analysis (HindIII and hindI). The sequences when compared with the one in the GeneBank showed 100% identity for TB-Q1, 99.6% for TB-Q2, 98.8% for D2, 99.6% for E2 and 100% for F2.

Discussion This study established a quick nested PCR protocol that could be finished within eight hours. It is sensitive enough to detect as minute DNA fragment as 1.35fg. Some studies reported similar results: for instance, PCR was sensitive enough to detect <10 organisms in 1ml of a milk sample pool (Sreevatsan *et al*, 2000). In other studies in bovine, it detected as few as one organism (5fg of DNA or less) (Floyd *et al*, 1992 and Vitale *et al*, 1998).

The IS1081 was used as a target for primer design because it occurs in many copies and it is very stable (van Soolingen *et al*, 1992). In a study in India, forty mycobacterial strains on restriction fragment length polymorphism analysis (RFLP) using IS1081 probes, showed the presence of 5 copies in all isolates of *M. bovis* and *M. tuberculosis* at the same chromosomal location (Sigh *et al*, 2004). In another USA study, nested PCR and PCR-REA assays were found to be 100% target specific with a concordance of 100% with pure cultures (n=200), 94.33% (100 of 103 samples) with acid fast smear positive sputum samples, 100% with CSF (15 of 15) and 62.5% with peripheral blood (Seyed *et al*, 2003).

However, other researchers reported IS1081 to bears a high degree of resemblance to the open reading frames of IS1245 and IS256 in *Staphylococcus aureus* (Colins *et al*, 1993 and Stanley *et al*, 1996). Therefore, we decided to test this PCR protocol with the DNA from; *Salmonella*, *Pasteurella multocida*, *Streptococcus*, *Actinobacillus pleuropneumoniae* and *Staphylococcus* for cross reactions, but all came out negative. This is in agreement with the results of another researcher, who tested One *M. bovis* BCG strain, two *M. tuberculosis* strains and ten species of mycobacteria other than tuberculosis (MOTT) with three PCR assays based on the repetitive elements IS6110, IS1081 and IS990 under variable amplification conditions (Dziadek *et al*, 2001). Amplification assays based on IS1081 and IS990 gave false-positive results in some MOTT isolates only under very low stringency (55°C), which could be due to non-specific priming of the target DNA at that temperature.

However, the mismatches in our study were sample number 78 and 198 (Table1). The explanation for 78 could be that the first sample got contaminated along the way from the point of collection to performing the PCR in the laboratory. Other researchers also cautioned that false-positive results may occur as a result of the cross-reactivity of PCR primers and probes or because of contamination during any stage of collection, processing, and detection (Sreevatsan *et al*, 2000), or it was a false negative due to an explanation which needs to be researched. It leaves us with a question as to whether a PCR positive animal remains positive or not for life. For the sample number 198, it could be that the pig was infected after we did the first examination or the infection dose was too low to be detected at the initial PCR test.

In this study, a positive PCR test indicates the presence of the pathogen in the pig, but a negative result does not rule out infection, since not all attempts to culture an organism are successful, and not all samples provide sufficient material to demonstrate positivity (Sreevatsan *et al*, 2000).

To further ascertain our developed PCR protocol REA and the PCR of recombinant plasmids showed the expected DNA bands. And all the cloned sequences had a high identity with the standard as follows: TB-Q1=100%, TB-Q2=99.6%, D2=98.8%, E2=99.6% and F2=100%.

Furthermore, there was need to differentiate *M. bovis* from *M. tuberculosis* for both epidemiological and public healthy reasons. The OxyR gene derived primers were found to be very effective for this purpose (Sreevatsan *et al*, 2000 and Espinosa *et al*, 1998).

Conclusions A rapid nested PCR protocol for the survey of *M. tuberculosis* and *M. bovis* in swine was developed and it's currently being applied by our research group to examine herds of swine for TB. In addition, another PCR protocol to differentiate *M. tuberculosis* and *M. bovis* when necessary was developed and demonstrated on reference strains. From our pilot research in Guangdong province, China, it appears the swine zoonotic TB is wide spread on some farms. A

large scale similar research could be carried out to establish the actual disease prevalence.

The cloning and sequencing results gave us an impression that our research methodology is of high quality.

Although PPD tests had low sensitivity and specificity compared to the nested PCR assay in this study, we recommend its use in an initial disease screening before nested PCR can be used.

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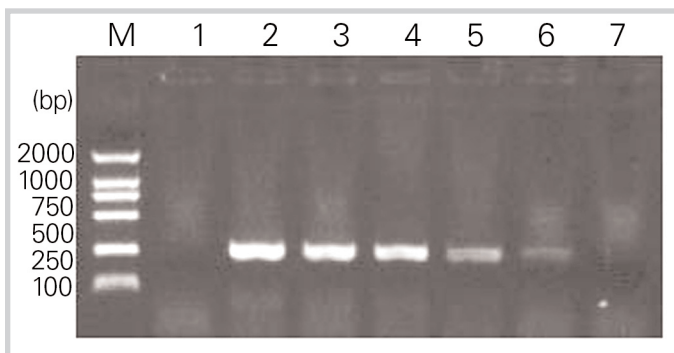


Fig1. Electrophoresis picture for nested PCR sensitivity test. M=DL2000 DNA Marker, 1=Negative control, 2=13.5pg, 3=1.35pg, 4=135fg, 5=13.5fg, 6=1.35fg, 7=0.135fg.

Sample numbers	Mark on the pig in the farm	1 st nested PCR results	PPD Skin test results	2 nd nested results
79	02-273002	+	-	-
504	02-247908	+	+/-	+
84	03-026602	+	+	+
100	KY01-1430	+	+/-	+
538	02-072310	+	+/-	+
95	03-246106	-	-	-
531	03-021904	-	-	-
127	03-029300	-	-	-
198	04-225402	-	+/-	+
568	SS01-19475	-	-	-

Table 1. Comparative between PCR and PPD tuberculin skin tests KEY: - there was no reaction (swelling, inflammation or nodule formation) on the animal. + the reaction was clear and positive. +/- there was a reaction but it was not very clear to be classified as positive or negative.